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GUIDELINES FOR DESIGNING PRIMERS

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University of Al-Qadisiyah
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GUIDELINES FOR DESIGNING PRIMERS

Proper primer design is important for applications in PCR, DNA sequencing, and hybridization. Here are some tips to help you design primers, especially using the Oligo program. This is based upon Oligo 4.0; there may be some changes compared to the current version.

A. General recommendations

The ideal primer generally has the following characteristics:

1. Melting temperature (T_m) between 55 and 65°C (usually corresponds to 45-55% G+C for a 20-mer).
2. Absence of dimerization capability.
3. Absence of significant hairpin formation (usually >3 bp).
4. Lack of secondary priming sites in the template.
5. Low specific binding at the 3' end, to avoid mispriming.

Usually a 20-24 nt primer works well. Oligo (as well as other programs) will pick decent primers automatically. However, often the "standard" parameters used by such programs don't work with a given sequence. In such a case, primers need to be picked manually. The following hints should help in this process:

B. Melting (and annealing) temperatures

As mentioned above, a T_m of 55-65°C works best in most applications. You may have noted that there are different ways to calculate T_m. The nearest neighbor thermodynamic calculation is the most accurate (make sure that you are looking at reaction conditions appropriate to your experiment (PCR, sequencing, etc.). Oligo will calculate T_m's for you (from the pull-down menus, select Analyze, then Composition and T_m):

```
Td = 50.3° [nearest neighbor method]
Tm = 62.2° [%GC method]
Tm = 52° [2°*(A+T) + 4°*(G+C) method]
```

For automated sequencing, primers with T_m's above 65-70°C can lead to secondary priming artifacts and noisy sequences. Remember, the sequence facility doesn't tailor the reactions to your specific primer as they use a "generic" annealing temperature.

Remember that you are dealing with TWO primers in PCR. Their T_m's should be within 5°C of each other; the closer the better! If T_m's are mismatched, amplification will

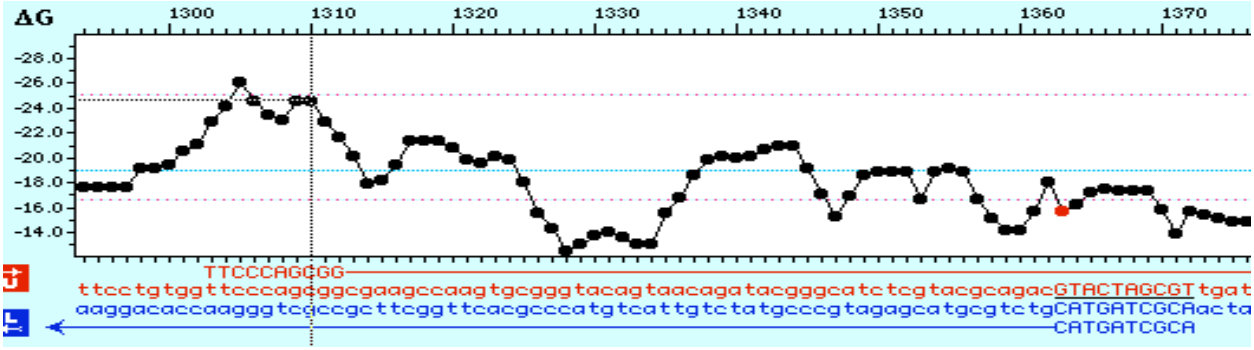
be inefficient: the primer with the higher T_m will misprime at lower temperatures, while the primer with the lower T_m may not work at higher temperatures. If the T_m difference is high, add or subtract bases from a primer. An easy way to see if two primers have similar T_ms using Oligo is to use the Analyze>DNA Amplification command in Oligo, which will show the T_ms of both primers at the same time. *Note that T_m is calculated at 50 mM salt, which is standard for PCR. If you were using labelled oligonucleotides for hybridizations in SSPE, you would need to calculate T_m differently (the Oligo program can also show a table that adjusts for differences in salt and formamide).*

```
Optimal Annealing Temperature:   56.6°
Product length, GC content & Tm: 84 bp, 58.3% GC, 75.8°
Product Tm - Upper Primer Tm:    14.2°
Primers Tm difference:           2.8°
Upper Primer: 22-mer, 5' pos. 1241, Tm = 61.6°, 3'-pentamer ΔG = -6.4 kcal/mol
Lower Primer: 20-mer, 3' pos. 1305, Tm = 64.4°, 3'-pentamer ΔG = -9.7 kcal/mol
Salt & DNA Concentrations (fixed in this option): 50 mM and 250 pM, respectively
```

The annealing temperature (T_a) for a primer pair is generally calculated as 5°C lower than the estimated melting temperature. The optimal temperature for PCR often needs to be determined empirically; ideally, the primers should anneal to the template before the template reanneals to itself. One way you can get into trouble designing primers is if you use AT-rich primers that flank a GC-rich region of DNA in PCR. Oligo will warn you of such problems, as well as suggest an optimal annealing temperature as shown above.

A special case in primer design (for PCR) is when you need to add extra bases to a primer, for example a restriction site. Typically one might design a primer that contains 18 nt perfectly matching the template, plus 6-nt representing the restriction site, and then about 2-nt more to assist in the restriction digestion (some enzymes need to "sit" upon a sequence larger than the restriction site itself; see the New England Biolabs catalog for more information). With this type of primer, there are essentially two different T_m's. Initially, the 18-nt region matching the template will define the T_m. After several rounds of PCR, most template will have incorporated the primer site, so the entire primer length will define the T_m. *For optimal results, one might consider doing a two-phase PCR program, shifting the T_m up by a few degrees after 5-10 cycles, however this may not really be necessary; consider doing this if the amplification appears to be working poorly.*

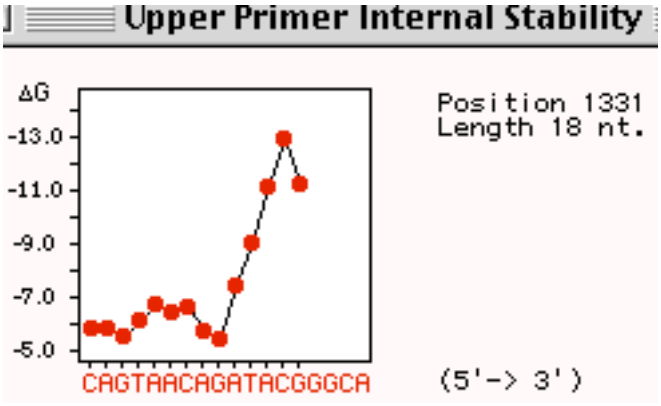
One way to start off with primers of similar T_ms is to use the Tm and ΔG windows in Oligo as a guide to select candidate primers. The scale at left indicates the ΔG of hybridization (this can be toggled to T_m if you wish), based on the size of the primer currently selected (press "L" to see that size) It is easy to see that the T_m of the left (upper) primer is going to be higher than that of the lower primer. Either the lower primer should be fitted to another region, or made longer.



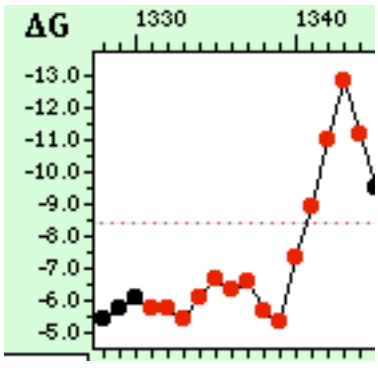
C. Specificity

Apart from T_m, a prime consideration in designing primers is ensuring that the likelihood of annealing to sequences other than the chosen target is very low. This can occur if the same sequence is present in the template DNA more than once, or when a primer is poorly designed.

To avoid mispriming, primers should not be very "sticky" on their 3' ends. A "sticky" 3' end would be one with a high G/C content (high T_m). Either the middle of the primer or the 5' end should be "stickier". The display in Oligo makes it easy to pick primers with good specificity, by using the "Internal Stability" window at the bottom of the screen or the Analyze>Internal Stability command (both are shown below). For example, the following primer (5'-CAGTAACAGA TACGGGCA) would show poor specificity since its 3' end is GC rich relative to the rest of the primer. NOTE that the issue is the 3' end region, not just the 3' base!!

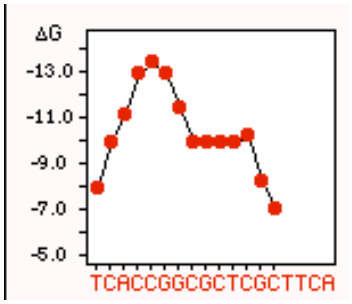


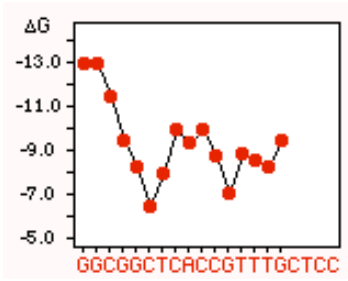
BAD!!!



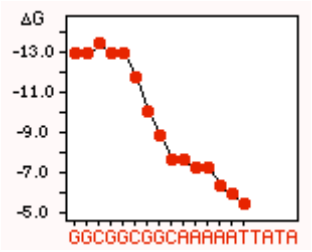
BAD!!!

In contrast, the following profiles are “good” for specificity (shown 5’—>3’), since the 3’ end is lower (in this ΔG plot) than the 5’ end or middle of the primers:





Now what about the profile shown below?



This would appear to give good specificity, based on the rules mentioned above. However, in practice this would be a difficult primer to use since at it’s T_m the 3’ end would anneal poorly to the template (remember that T_m is the 50% point in a melting curve). This brings up the “rule of extremes” in primer design:

Avoid primers with long polyG or polyC stretches that can promote non-specific annealing.
Avoid polyA and polyT as these will “breath” and open the primer-template complex.
Avoid polypyrimidine (T, C) and polypurine (A, G) stretches, which may lead to an odd shape of the double helix.
Ideally the primer will have a near random mix of nucleotides.

D. What about having a “GC clamp” at the 3’ end?

A "G" or "C" is desirable at the 3' end of primers since this will reduce “breathing” and thereby increase yield. However, Gs or Cs should not be added if they adversely influence the overall specificity of the primer!

E. Primer-Primer interactions

When designing primers, it is important to have a minimum of intramolecular or intermolecular homology. This would result in either hairpins or primer dimerization. If a primer has a region of self-homology, “snap back” or partially double-stranded

structures can occur which will interfere with annealing to the template. Usually intraprimer homologies of 3 bp or more should be avoided. The resulting hairpins are readily detected in Oligo using the Analyze>Duplex (or Hairpin) command, for example:

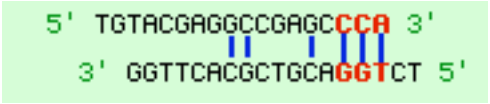
Primers should also not contain sequences of nucleotides that would allow one primer molecule to anneal to another molecule of itself, or to the other primer (if being used in PCR). Such interactions can also readily detected in Oligo using the Analyze>Duplex command (remember to check both the individual primer and upper/lower primer combinations). Also, remember that primer-primer interactions are stronger at lower temperatures; a small degree of complementarity becomes less significant as reaction temperatures increase *(in other words, something that is OK with an annealing temperature of 60C may work poorly at an annealing temperature of 50C).*

The worst situation is when the 3' ends of the primer(s) anneal; this leads to "primer-dimer" formation:

Internal intermolecular interactions should also be minimized. Oligo provides a ΔG value for such complexes. Ideally, the smallest ΔG, the better but try to avoid primers annealing with ΔG values of –7 kcal/mol or higher. Having a complementary TA sequence at the 3' ends of primers usually doesn't cause problems since it is not very stable, however a complementary GC region can cause problems at T_m's below 65C. The primer shown below has a T_m of only 50°C, so the 3' GC homology (ΔG=-3.1 kcal/mole) might be a problem; if the T_m was more like 60°C, then the primer would probably be OK.

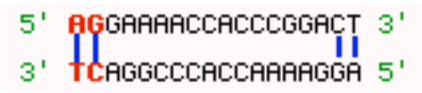
Here are other examples of interacting primers. The following are OK, but not great:

ΔG=-5 kcal/mole, OK but not great.



ΔG=-9.3 kcal/mole, pretty bad!!

The next is real nice! ΔG=-1.6 kcal/mole, good!!



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